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Whole-Genome Comparison of *Aspergillus fumigatus* Strains Serially Isolated from Patients with Aspergillosis

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The emergence of azole-resistant strains of *Aspergillus fumigatus* during treatment for aspergillosis occurs by a mutation selection process. Understanding how antifungal resistance mechanisms evolve in the host environment during infection is of great clinical importance and biological interest. Here, we used next-generation sequencing (NGS) to identify mutations that arose during infection by *A. fumigatus* strains sequentially isolated from two patients, one with invasive pulmonary aspergillosis (IPA) (five isolations) and the other with aspergilloma (three isolations). The serial isolates had identical microsatellite types, but their growth rates and conidia production levels were dissimilar. A whole-genome comparison showed that three of the five isolates from the IPA patient carried a mutation, while 22 mutations, including six nonsynonymous ones, were found among three isolates from the aspergilloma patient. One aspergilloma isolate carried the *cyp51A* mutation P216L, which is reported to confer azole resistance, and it displayed an MIC indicating resistance to itraconazole. This isolate harbored five other nonsynonymous mutations, some of which were found in the *afyap1* and *aldA* genes. We further identified a large deletion in the aspergilloma isolate in a region containing 11 genes. This finding suggested the possibility that genomic deletions can occur during chronic infection with *A. fumigatus*. Overall, our results revealed dynamic alterations that occur in the *A. fumigatus* genome within its host during infection and treatment.

Aspergillus fumigatus is a major pathogen responsible for aspergillosis, a disease that causes invasive pulmonary aspergillosis (IPA), pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis. This fungus usually infects only immunocompromised patients, in whom it has a relatively high mortality rate when causing IPA (1, 2). The treatment of aspergillosis has proven difficult because of limited antifungal therapy possibilities and the prevalence of antifungal-resistant strains worldwide. Consequently, the number of cases for which treatment has failed is increasing (3, 4). Recent studies from several research groups show that prolonged azole therapy tends to promote the occurrence of azole-resistant strains (5, 6). However, long-term antifungal therapy is indispensable for the treatment of chronic aspergillosis. A further understanding of how antifungal-resistant mechanisms evolve in the host environment during infection or colonization is therefore of great clinical importance and biological interest.

Azole drugs are often used as a first-line therapy for aspergillosis (7). Itraconazole (ITCZ) can treat chronic aspergillosis infections, such as aspergilloma, whereas voriconazole (VRCZ) is used to treat invasive aspergillosis. Since first being reported in 1997, a number of azole-resistant strains have been isolated primarily from aspergilloma patients on prolonged azole therapy (4, 8–10). Fungal tolerance is frequently caused by mutations in the gene encoding the target protein of azoles (*cyp51A*). Several mutations in *cyp51A* have been found in azole-resistant strains, and amino acid substitutions in the Cyp51A protein, including G54A, G54W, P216L, M220V/K/T, and G448S, have been shown to cause azole tolerance in genetic reconstitution experiments (5, 11, 12).

Generally, most mutations causing azole resistance are thought to have been generated during infection in an azole-treated host (3, 5, 6). In some cases in which the strains are serially isolated from a single patient undergoing azole treatment, the first cultured isolates show azole sensitivity and carry no *cyp51A* mutations, whereas subsequent isolates are azole resistant and possess *cyp51A* mutations (3, 5, 6, 13). Although it is not clear whether the host environment itself promotes genetic mutations, azole treatment appears to be a selective factor for *cyp51A* mutants.

In recent years, several genotyping methods, such as multilocus sequence typing (MLST) and microsatellite analysis of short tandem repeats (STRs), have been developed for the genetic discrimination of clinical and environmental *A. fumigatus* strains (14, 15). Intensive genotyping studies have shown that clinical isolates recovered from a single patient with aspergilloma sometimes contain different genotypes (6, 16, 17). In a recent study by Howard et al. (18), strains recovered from surgically removed fungal balls that consisted of masses of mycelia and resided in the lungs of aspergilloma patients were used for genetic typing. Microsatellite variation, the coexistence of strains with different genotypes, and different azole susceptibilities were found among the strains from the same fungal ball. This clearly shows that a mature fungal ball consists of genotypically and/or phenotypically distinct strains, which have evolved during persistence in the host lung.

Next-generation sequencing (NGS) is becoming increasingly affordable. It enables a whole-genome sequence (WGS)-based comparison to be performed between clinical isolates, thus pro-

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TABLE 1 Isolates used in this study

Isolate by patient	Date of isolation (day/mo/yr)	No. of tandem repeats at microsatellite site								
		2A	2B	2C	3A	3B	3C	4A	4B	4C
I (IPA)										
IFM 59355-1	21/07/2009	20	16	14	8	17	23	5	11	10
IFM 59355-2	21/07/2009	20	16	14	8	17	23	5	11	10
IFM 59356-1	24/07/2009	20	16	14	8	17	23	5	11	10
IFM 59356-2	24/07/2009	20	16	14	8	17	23	5	11	10
IFM 59356-3	24/07/2009	20	16	14	8	17	23	5	11	10
II (aspergilloma)										
IFM 59361-1	09/11/2009	24	19	14	50	14	29	10	7	9
IFM 59361-2	09/11/2009	24	19	14	50	14	29	10	7	9
IFM 60237	10/03/2011	24	19	14	51	14	29	10	7	9

viding high-resolution typing. Using this method, Camps et al. (19) uncovered that a novel mutation in the *hapE* gene encoding a CCAAT-binding transcription factor complex subunit had occurred during infection and was responsible for azole resistance. This study highlighted the potential for NGS to reveal unknown mechanisms for azole resistance.

In the present study, sequential isolations of *A. fumigatus* from one patient with IPA and another with aspergilloma were subjected to WGS analysis in order to better understand the genomic dynamics of this pathogen during infection and treatment. The WGS comparison revealed several punctual mutations and a large-segment deletion among different strains. To the best of our knowledge, this is the first report of genomic rearrangement during infection in pathogens responsible for causing aspergillosis.

MATERIALS AND METHODS

Strains. The *A. fumigatus* clinical strains analyzed here were isolated at Chiba University Hospital, Japan. Five isolates were collected from patient I, who had IPA, on different days (21 and 24 July 2009). From patient II with aspergilloma, three isolates were collected on different days (9 November 2009 and 10 March 2011) (Table 1). The isolates were morphologically, physiologically, and genetically identified as *A. fumigatus* and were maintained by the Medical Mycology Research Center (MMRC) of Chiba University. For distinction, the isolates obtained on the same day were named as follows: IFM 59355-1 and IFM 59355-2; IFM 59356-1, IFM 59356-2, and IFM 59356-3; and IFM59361-1 and IFM 59361-2. After the present study, we renamed the strains IFM 62541 (for IFM 59355-1), IFM 62542 (for IFM 59355-2), IFM 62543 (for IFM 59356-1), IFM 62544 (for IFM 59356-2), IFM 62545 (for IFM 59356-3), IFM 62546 (for IFM 59361-1), and IFM 62547 (for IFM 59361-2). All isolates were preserved at the MMRC.

IFM 59355-1, IFM 59355-2, IFM 59356-1, IFM 59356-2, and IFM 59356-3 were isolated from a patient with IPA, as defined by the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria (patient I) (20). IFM 59361-1, IFM 59361-2, and IFM 60237 were isolated from a patient radiologically diagnosed with aspergilloma in 2007 (patient II), as defined by the criteria suggested by Denning (21) (Table 1). IFM 60237 was isolated 16 months after the other two strains. All isolates were obtained by culturing patient sputum samples.

Patient I was treated with voriconazole after the isolation of the strains studied here (around 24 July 2009); therefore, the isolates from this patient were not exposed to azole drugs preisolation. Patient II was treated with voriconazole (VRCZ) (400 mg daily) in December 2009 (1 month after the first isolation of *A. fumigatus*), but the treatment was discontinued after 5 days because of a side effect. Three days later, itraconazole (ITCZ) treatment was started (200 mg daily) and lasted for 20 months.

Microsatellite genotyping. Nine microsatellite regions of approximately 400 bp were PCR amplified using appropriately designed primer pairs (see Table S1 in the supplemental material) and sequenced using Sanger sequencing (14). The sequences of the primers are shown in Table S1. The repeat numbers of each locus were counted from the sequences.

Growth and conidiation tests. First, conidia from each isolate were obtained from the MMRC stock center (as lyophilized conidia). To prepare fresh conidial suspensions, well-segregated conidia were inoculated onto a potato dextrose agar (PDA) slant and incubated at 37°C for 7 days. An appropriate volume of phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 (PBST) was added and vortexed gently to obtain conidial suspensions. To eliminate hyphae, the conidial suspension was passed through a cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). To test for colony growth, 10^4 conidia of each strain were point inoculated onto glucose minimal medium (GMM) (22) or PDA plates, which were incubated at 37°C for 66 h or 48 h, respectively.

A calculation of the number of conidia obtained was performed as described previously (23). Briefly, conidia on PDA (10^4 conidia/ml, 3 ml per well) were incubated at 37°C in 6-well plates. After 48 h of incubation, the agar from each well containing the conidia (including mycelia) was vortexed in 10 ml of PBST. The numbers of conidia in the suspensions were counted using a hemocytometer. The data were obtained in triplicate, and the mean values ± standard deviations (SD) were reported.

Whole-genome sequencing. Genomic DNA was prepared from overnight cultured mycelia using the DNeasy plant minikit (Qiagen, Hilden, Germany). The preparation of a fragmented genomic DNA library was performed using a Nextera DNA sample preparation kit, according to the manufacturer's protocols (Illumina, San Diego, CA). We prepared the DNA libraries for each strain in our laboratory. The mean length of the libraries ranged from 400 to 1,500 bp (see Tables S2 and S3 in the supplemental material). Sequencing was conducted in a paired-end 2 × 150 bp mode on a MiSeq system, according to the manufacturer's protocols (Illumina).

Sequence analysis. The Illumina data sets were trimmed using fastq-mcf in ea-utils (version 1.1.2-484), i.e., sequencing adapters and sequences with low quality scores (Phred score [Q], <30) were removed (24). The data sets were mapped to the genome sequence of the *A. fumigatus* genome reference strain Af293 (29,420,142 bp, genome version s03-m04-r03) (25, 26) using Bowtie 2 (version 2.0.0-beta7) with the very sensitive option in end-to-end mode (27). Duplicated reads were removed using Picard (version 1.112) (http://picard.sourceforge.net). The programs mpileup and bcftools from SAMtools (version 0.1.19-44428cd) were used to perform further quality controls. In mpileup, the -q20 argument was used to trim reads with low-quality mapping, whereas the argument -q30 was used to trim low-quality bases at the 3′ end (28). The bcftools setting was set to -c in order to call variants using Bayesian inference. Consensus and single nucleotide polymorphisms (SNPs) were excluded if they did not meet a minimum coverage of 5× or if the variant was present in <90% of the base calls (29, 30). The genotype field in the variant call format (VCF) files indicates homozygote and heterozygote probabilities as Phred-scaled likelihoods. SNPs were excluded if they were called as heterozygous genotypes using SAMtools. The mapping results were visualized in the Integrative Genomics Viewer (version 2.3.3) (31, 32). The reference genome data included information on open reading frames and annotations, from which the SNPs were designated nonsynonymous or synonymous.

Single nucleotide mutations were confirmed by Sanger sequencing. Regions of approximately 400 bp that contained a mutation were amplified with appropriately designed primer pairs and then sequenced. The primer sequences are listed in Table S1 in the supplemental material, which were named as follows. For verification of the SNPs in strains from patient I or patient II, PaI or PaII was added to the primer name, respectively. For nonsynonymous SNPs, synonymous SNPs, or SNPs in a noncoding region, "(NS)," "(Syno)," or "(NonC)" was added to the primer name, respectively.

Analysis of unmapped reads. *De novo* assembly of the unmapped reads was conducted using the Newbler assembler 2.9 (Roche), with de-

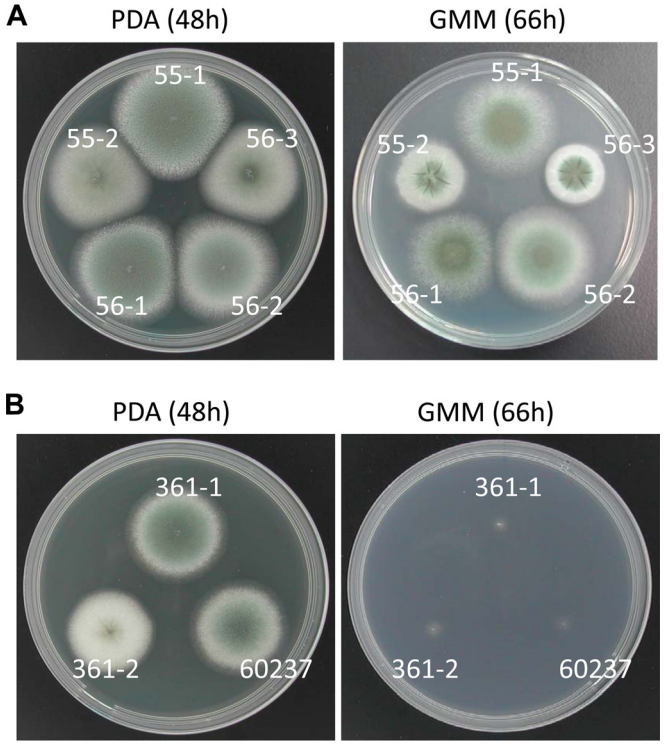

FIG 1 Comparison of colonial growth among the isolates. The conidia (10^4) of the isolates from patient I (A) or patient II (B) were point inoculated onto glucose minimal medium (GMM) or potato dextrose agar (PDA) plates and then incubated at 37°C for 66 h or 48 h, respectively.

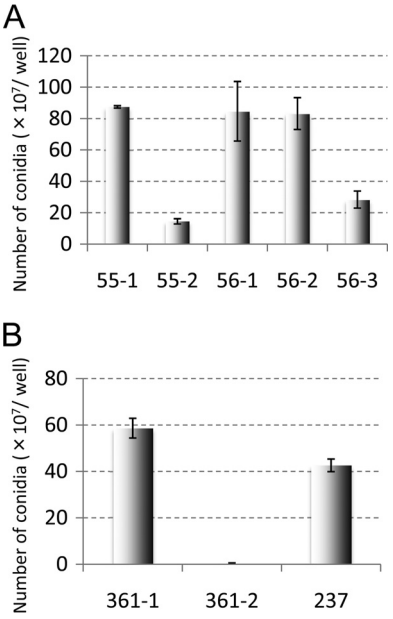

FIG 2 Comparison of conidia numbers among the isolates. Three milliliters of PDA containing 3×10^4 conidia was incubated at 37°C in each well of a 6-well plate. After 48 h, the agar was vigorously vortexed in 10 ml of PBS containing 0.1% Tween 20. The conidia numbers were counted using a hemocytometer. The data were obtained in triplicate, and mean values ± SD are shown.

fault parameters. The contigs were selected based on size/depth criteria: those of <500 bp and/or with a depth of <30× coverage were removed. To investigate whether unique genome sequences were present in strains isolated from the same patient, the unmapped reads of each strain were mapped to the contigs generated from all the strains in the same patient by the Bowtie 2 software. The coverage of the mapped regions was then evaluated. Gene predictions were performed using the gene prediction tool AUGUSTUS (version 2.5.5), with a training set of *A. fumigatus* (33). The parameters of AUGUSTUS were -species = aspergillus_fumigatus, -strand = both, -genemodel = partial, -singlestrand = false, -protein = on, -introns = on, -start = on, -stop = on, -cds = on, and -gff3 = on. To compare all the predicted genes with *Aspergillus* genes, consisting of 244,811 genes available on AspGD (34), a reciprocal BLAST best hit approach was performed by BLASTp (35), with an E value of 1.0e−4. All BLASTp results were filtered based on a BLASTp identity of ≥80% and an aligned length coverage of ≥80%.

Antifungal susceptibility testing. Antifungal susceptibility tests were performed as described previously (36). Briefly, tests were performed in triplicate using amphotericin B (AMPH-B), 5-flucytosine (5-FC), fluconazole (FLCZ), ITCZ, VRCZ, miconazole (MCZ), and micafungin (MCFG) in RPMI 1640 medium (pH 7.0) at 35°C, according to the Clinical and Laboratory Standards Institute reference broth microdilution method, document M38-A2 (37), with partial modifications using the dried plate for antifungal susceptibility testing of yeasts method (Eiken Chemicals, Tokyo, Japan).

Accession number. The sequenced genome data were deposited with the DDBJ Sequence Read Archive under accession no. DRA002475.

RESULTS

Microsatellite analysis of the isolates from patients I and II. To determine whether the isolates from each patient were genotypically identical, we calculated the number of STRs in the microsatellites, as described in a report by de Valk et al. (14). The five isolates from patient I showed identical repeat numbers at all loci, indicating an identical genotype. In the three isolates from patient II, however, IFM 60237 had a different repeat number for locus 3A, whereas the other loci were the same as those of IFM 59361-1 and IFM 59361-2. Because the locus 3A difference amounted to only one repeat, we concluded that the strains were originally identical.

Phenotypic analysis of the isolates. Of the five isolates from patient I, IFM 59355-2 and IFM 59356-3 had retarded growth on GMM and relatively white colonies on GMM and PDA plates. Furthermore, the surface patterns of the isolates on GMM differed from each other (Fig. 1A). In contrast, the growth of the isolates from patient II on GMM was almost completely impaired. On PDA, IFM 59361-2 produced a white colony, whereas the other two strains each produced a greenish colony (Fig. 1B).

Of the isolates from patient I, IFM 59355-2 and IFM 59356-3 showed a 70 to 80% reduction in conidia production compared with that of the other isolates (Fig. 2A). Of the isolates from patient II, conidia production in IFM 59361-2 decreased to 1% of that in IFM 59361-1, whereas IFM 60237 showed a 27% decrease compared with that of IFM 59361-1 (Fig. 2B). Collectively, these strains exhibited different phenotypes from each other despite originally being from an identical set from each patient.

Whole-genome sequence comparisons. To gain insight into the phenotypic differences among the strains isolated from patients I and II, a WGS-based comparison was conducted using NGS. For five strains from patient I, the mapped sequences of IFM 59355-1, IFM 59355-2, IFM 59356-1, IFM 59356-2, and IFM 59356-3 were 27,086,733, 27,086,107, 27,106,829, 26,501,391, and 26,925,829 bp, respectively. Similarly, for three strains from pa-

TABLE 2 Mutations detected in isolates from patient I

Gene	Amino acid length	Description	Amino acid substitution				
			IFM 59355-1	IFM 59355-2	IFM 59356-1	IFM 59356-2	IFM 59356-3
Afu1g12490	570	Sexual development regulator (*veA*)		Gln 400[a]			
Afu2g08040	1,148	C6 finger domain protein, putative	Tyr 958 Cys				
Afu8g06170	270	Hypothetical protein				Thr 260 Asn	

[a] Nonsense substitution.

tient II, the mapped sequences of IFM 59361-1, IFM 59361-2, and IFM 60237 were 27,206,287, 27,205,914, and 26,803,686 bp, respectively. The rates of the mapped region in the reference genome (Af293, 29.4 Mb) ranged from 90.1 to 92.5%, and the total coverages of the mapping ranged from 15.0 to 45.8× (see Tables S2 and S3 in the supplemental material).

Among the regions mapped by all five strains from patient I or all three strains from patient II, four or 22 loci were estimated to be an SNP, respectively. These candidate loci were confirmed by Sanger sequencing, although one of the loci found in the patient I strain set was a false call. The other three SNPs from the patient I strains were valid and identified as nonsynonymous (Table 2). IFM 59355-2 has a nonsense mutation in the *veA* gene at Q400, while IFM 59355-1 and IFM 59356-2 have a substitution in Afu2g08040 (Y958C) and Afu8g06170 (T260N), respectively. No SNPs were detected in the IFM 59356-1 or IFM 59356-3 genomes.

Among the strains from patient II, 22 SNPs were identified, of which six were nonsynonymous (Table 3, E478K in Afu2g15890, P216L in *cyp51A*, F487L in *afyap1*, G357S in *aldA*, Q1047 in Afu7g04400, and T397N in Afu8g07080). Notably, all of these SNPs were found in IFM 60237, which was isolated 16 months after the other two strains. Moreover, one synonymous SNP was identified in the Afu7g05660 locus of IFM 60237 (Table 3). The other 15 SNPs were located in noncoding regions, such as untranslated regions, introns, and intergenic regions (Table 4). The locations of these SNPs were not limited in IFM 60237, which was in contrast to the case of the nonsynonymous SNPs, and it suggested that the noncoding regions may be more variable.

Because we used the Af293 genome sequence as a reference for mapping analysis, it is conceivable that we missed genomic regions unique to the strains. Therefore, to explore these unique regions from each patient, we assembled the unmapped reads to construct contigs for the strains from each patient. A total of 205 and 474 contigs were generated, with total lengths of 767,194 and 1,552,135 bp for patients I and II, respectively (Table 5). We next used the gene prediction tool AUGUSTUS to identify the coding sequences from each contig. A total of 163 and 396 genes were predicted from each of the strains from patients I or II, respectively (Table 5). We then compared the gene contents of the unique regions between strains from the same patient, revealing no differences in the unmapped regions. Although we could not determine differences at the SNP level in the unique regions because of technical difficulties, these results suggest that those genomic regions absent from the Af293 strain might possess a similar structure in strains isolated from the same patient.

To compare the gene contents in regions common to the Af293 strain, we explored draft genome sequences. By searching for the loss of coding regions among the strains from each patient, we found that a region of approximately 38.5 kb containing 11 genes was missing in the IFM 60237 genome compared with the IFM 59361-1 and IFM 59361-2 genomes from patient II (Table 6); no large-scale deletion was found among the strains from patient I. Collectively, we identified several genetic differences (SNPs and a large-scale deletion) among the serially isolated strains using a WGS comparison, although no mutations that might affect phenotypes were identified in the IFM 59356-1 and IFM 59356-3 genomes.

Acquired tolerance to ITCZ in a strain from an aspergilloma patient. WGS comparisons revealed that IFM 60237 carried a *cyp51A* mutation compared with the genomes of the other two strains. This substitution (P216L) was previously shown to confer ITCZ resistance; therefore, we assumed that IFM 60237 was resistant to some azole drugs (5). The antimicrobial susceptibilities of the strains from both patients were determined by testing with MCFG, AMPH-B, and 5-FC, as well as the azole drugs FLCZ, ITCZ, VRCZ, and MCZ (Table 7). As expected, IFM 60237 had an MIC indicative of an ITCZ-resistant phenotype (4 mg/liter). The ITCZ MICs of the other strains showed an ITCZ-susceptible phenotype, which is consistent with the absence of the *cyp51A* P216L substitution. The susceptibilities to other drugs were similar between each set of strains.

VRCZ and ITCZ had been used to treat patient II for aspergil-

TABLE 3 Nonsynonymous and synonymous mutations detected in isolates from patient II

Gene	Amino acid length	Description	Amino acid substitution		
			IFM 59361-1	IFM 59361-2	IFM 60237
Afu2g15890	772	RING finger protein, putative			Glu 478 Lys
Afu4g06890	515	14-Alpha sterol demethylase (*cyp51A*)			Pro 216 Leu
Afu6g09930	615	bZip family transcription factor (*afyap1*)			Phe 487 Leu
Afu6g11430	559	Putative aldehyde dehydrogenase (*aldA*)			Gly 357 Ser
Afu7g04400	1,220	Hypothetical protein			Gln 1047[a]
Afu7g05660	1,065	Hypothetical protein			Thr 1018 Thr
Afu8g07080	634	Putative secreted metalloprotease			Thr 397 Asn

[a] Nonsense substitution.

TABLE 4 Mutations detected in noncoding regions of isolates from patient II

Chromosome	Position	Region	Related gene or proximal gene	Nucleotide substitution in isolate:			Reference nucleotide
				IFM 59361-1	IFM 59361-2	IFM 60237	
1	3052529	5′ UTR[a]	Afu1g11560	G	G	—[b]	A
	3288510	5′ UTR	Afu1g12480	—	—	T	G
2	4499618	3′ UTR	Afu2g16840	—	—	T	G
3	545649	Intergenic		C	—	—	G
	1446159	Intron	Afu3g05900	—	—	T	G
4	1137864	5′ UTR	Afu4g04060	—	—	T	C
	1682647	Intergenic		T	T	—	T
	2221124	Intergenic		—	—	C	G
	2625031	Intergenic		—	—	A	G
5	489586	3′ UTR	Afu5g01920	—	—	T	C
	1779941	Intergenic		A	A	—	C
	3582922	Intergenic		T	T	—	C
6	1473248	5′ UTR	Afu6g06740	C	C	—	T
	2286061	Intergenic		A	A	—	C
8	928033	Intergenic		—	—	T	C

[a] UTR, untranslated region.
[b] —, nucleotide identical to the reference.

loma (Fig. 3). Before isolation, IFM 60237 had been exposed to 5 days of VRCZ and 449 days of ITCZ. This duration of drug exposure might have led to the development of drug resistance, namely, that conferred by *cyp51A* P216L. Patient I had never been treated with azoles before the strains were isolated.

DISCUSSION

We analyzed three strains serially isolated from an aspergilloma patient (patient II) to compare their draft genome sequences and phenotypes. While the strains originally contained identical microsatellites (although IFM 60237 exhibited microevolution), their conidia production levels on PDA plates differed markedly from each other (Fig. 1 and 2). Because genetic differences, such as nonsynonymous mutations and deletions among these strains, were only found in the IFM 60237 genome, the reason for the marked decrease in conidiation in IFM 59361-2 is unknown.

One possible explanation is that we investigated only SNPs in regions that mapped with at least five reads. For example, a 26.8- to 27.2-Mb region corresponded to 91.1 to 92.5% of the reference genome (29.4 Mb); therefore, if the strains have genomes comparable in size to that of Af293, this would leave a 2.2- to 2.6-Mb region uninvestigated. In fact, compared with Af293, another sequenced *A. fumigatus* strain, A1163, has up to 2% unique genes in its genome (38), which might explain the phenotypic differences. To test this possibility, we constructed and explored the unique regions absent from the Af293 genome in patients I and II, predicting 163 and 396 genes corresponding to 1.7% and 4.0% of the Af293 genome, respectively. Nonetheless, the gene contents in these regions were identical among the strains of each patient. Another possible explanation is that mutations in noncoding regions might be responsible for the phenotypic differences. Although such mutations will not affect a protein sequence directly, the rates of transcription, mRNA processing, and translation might be influenced. We identified 15 SNPs in noncoding regions in IFM 59361-1, IFM 59361-2, and IFM 60237 (Table 4). However, the specific effects of these SNPs on the phenotypes are yet to be investigated.

IFM 60237 was recovered 16 months after the isolation of IFM 59361-1 and IFM 59361-2 from patient II and was shown to carry a mutation in *cyp51A* known to be involved in azole resistance (5). This isolate subsequently showed a high ITCZ MIC, revealing its resistance to ITCZ. This is likely to have been caused by the 16-month exposure to azole treatment inside the host, which is consistent with previous reports that showed that resistance mutations were generated during azole treatment (3, 5, 6).

Besides the *cyp51A* mutation, the WGS comparison provided evidence for several SNPs in other genes and noncoding regions in the strains from the aspergilloma patient (Tables 3 and 4). Because most aspergillosis studies comparing serial isolates have focused on *cyp51A* mutations involved in azole resistance, genome-wide information about mutations occurring during infection is limited (5). Thus, the finding in this study offers a genome-wide view that genomic alterations at the SNP level are a dynamic process occurring during aspergilloma persistence. For pathogenic bacteria, several WGS-based studies have been published that uncovered mutations that arose during persistence within the host (39–41). The WGS comparison approach has been increasingly applied to advance our understanding of bacterial genomic diversification during infection. In contrast, few WGS-based studies have been carried out for pathogenic fungi. The only such study recently published identified five loci that showed genetic variation between the genomes of two *Cryptococcus neoformans* strains serially isolated 77 days apart (42). One of these five mutations was associated with phenotypic differences in virulence factors, indicating that the WGS comparison is a powerful tool to investigate intraclonal isolates of pathogenic fungi.

TABLE 5 Summary of *de novo* assembly of unmapped reads

Assembly parameter	Patient I (5 strains)	Patient II (3 strains)
No. of contigs (length ≥ 500, depth ≥ 30)	205	474
Total length (bp)	767,194	1,552,135
N_{50} (bp)	11,069	7,531
Avg length (bp)	3,742	3,274
No. of predicted genes	163	396

TABLE 6 List of genes absent in the IFM 60237 genome

Gene	Description	Presence/absence in isolate:		
		IFM 59361-1	IFM 59361-2	IFM 60237
Afu7g06780	Hypothetical protein	Present	Present	–[a]
Afu7g06790	Putative allantoate permease of the major facilitator superfamily	Present	Present	–
Afu7g06800	Glutamyl-tRNA (Gln) amidotransferase, subunit A	Present	Present	–
Afu7g06810	L-Amino acid oxidase LaoA, putative	Present	Present	–
Afu7g06820	Hypothetical protein	Present	Present	–
Afu7g06825	rRNA	Present	Present	–
Afu7g06830	Hypothetical protein	Present	Present	–
Afu7g06840	Putative class III aminotransferase	Present	Present	–
Afu7g06850	Phosphoserine phosphatase, putative	Present	Present	–
Afu7g06890	Peroxidase, putative	Present	Present	–
Afu7g06900	Putative branched-chain amino acid aminotransferase protein	Present	Present	–

[a] –, absent.

The WGS comparison in this study uncovered not only genetic mutations but also a large-scale deletion (38.5 kb) in chromosome 7 of IFM 60237 (Table 6). We found 11 missing genes compared to the genomes of other strains, although the effects of the deletion on the fungal phenotype remain to be determined. Pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, have been shown to acquire large deletions in their genomes during chronic infection (39, 41). Indeed, some bacterial pathogens appear to establish relatively long-term infections that may contribute to such genomic deletions. In the case of aspergilloma, fungal balls can reside asymptomatically in host lung cavities for years. The present study suggests that genomic deletions can also occur during chronic infection in pathogenic fungi, as observed in bacteria. Together, these findings prompt us to speculate that a mature fungal ball consists of genotypically identical strains that randomly accumulate mutations and genomic deletions. To verify this hypothesis, WGS comparisons should be made using larger numbers of strains recovered from one patient with aspergilloma. Although our collection of aspergilloma clinical isolates was insufficient for this purpose at the time this study was conducted, a further study is planned.

Three nonsynonymous mutations were identified from IPA isolates (Table 2). One of these in IFM 59355-2 *veA* was a nonsense mutation at position 400. Although it has been reported that an *A. fumigatus veA*-null mutant showed hyperactivation of conidiation, it is unclear if the truncated protein retained full functionality and, therefore, if decreased conidiation in this strain was associated with this mutation (43). No information is currently available about the proteins encoded by Afu2g08040 and Afu8g06170.

Six nonsynonymous mutations were found in IFM 60237 (Table 3). As stated above, the mutation in *cyp51A* is most likely responsible for azole resistance. The *afyap1* gene encodes an oxidative stress-responsive transcriptional regulator highly conserved among fungi (44, 45). The AfYap1 protein has been reported to play a crucial role in oxidative stress tolerance but not in virulence (46). We observed an amino acid substitution (F487L) of Afyap1 in the IFM 60237 genome, which appears to be located outside the functional domains, based on conserved motif analysis. Investigations into the oxidative stress sensitivities of these strains found that they were indistinguishable in their responses to hydrogen peroxide, suggesting that the mutation in *afyap1* is not related to an oxidative stress function (data not shown). During infection, pathogens are exposed to several types of stress from the host environment, including high temperature, low nutrition, hypoxia, iron deficiency, and immune responses; such stresses may promote spontaneous mutations in *A. fumigatus* during infection.

In conclusion, the WGS comparisons in the present study

TABLE 7 MICs for the *A. fumigatus* isolates used in this study

Isolate by patient	MEC for MCFG (mg/liter)[b]	MIC for (mg/liter)[c]:					
		AMPH-B	5-FC	FLCZ	ITCZ	VRCZ	MCZ
I (IPA)[a]							
IFM 59355-1	0.03	2	16	>64	0.5	1	2
IFM 59355-2	0.03	2	32	>64	0.5	0.5	2
IFM 59356-1	0.03	2	2	16	0.5	0.5	1
IFM 59356-2	0.03	1	32	>64	0.5	0.5	2
IFM 59356-3	0.03	2	4	>64	1	0.5	1
II (aspergilloma)							
IFM 59361-1	0.03	1	4	8	1	1	1
IFM 59361-2	0.03	1	8	>64	1	2	2
IFM 60237	≤0.015	2	8	64	4	1	0.5

[a] IPA, invasive pulmonary aspergillosis.
[b] MCFG, micafungin; MEC, minimum effective concentration.
[c] AMPH-B, amphotericin B; 5-FC, 5-flucytosine; FLCZ, fluconazole; ITCZ, itraconazole; VRCZ, voriconazole; MCZ, miconazole.

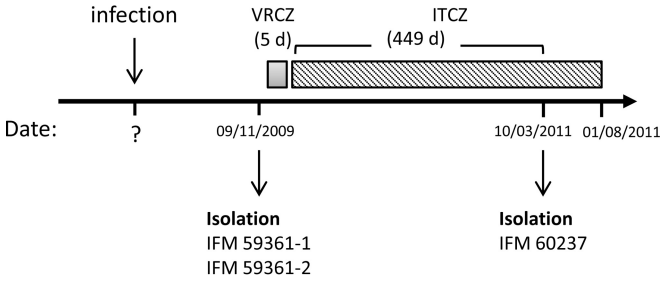

FIG 3 Treatment schedule for patient II. The drug treatments are indicated by bars. The corresponding fungal isolation dates are shown. In this patient, a fungal ball was observed radiologically in 2007; therefore, the causal infection supposedly occurred before this date. The dates are shown in day/month/year format. d, days.

identified several genetic alterations in a set of serially isolated clinical *A. fumigatus* strains. The NGS analysis proved useful in discovering genomic differences between intraclonal strains that would not have been detectable using existing methods, such as MLST and microsatellite detection. In fact, our NGS analysis presented the possibility that genomic deletions occur during chronic infection in pathogenic fungi. This successful resolution paves the way for understanding dynamic alterations that occur in a fungal pathogen genome within its host, as well as how the host environment can alter phenotypic properties.

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